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Award Number: W81XWH-06-1-0407

TITLE: Cadmium, Zinc, and Selenium Levels in Carcinoma of the Human Prostate

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REPORT DATE: April 2008

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
01-04-2008

2. REPORT TYPE
Final

3. DATES COVERED
15 Mar 2006 – 14 Mar 2008

4. TITLE AND SUBTITLE

Cadmium, Zinc, and Selenium Levels in Carcinoma of the Human Prostate

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0407

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

American Registry of Pathology
Armed Forces Institute of Pathology
Washington, DC 20306

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT The purpose of this study is to examine associations of zinc (Zn), selenium (Se) and cadmium (Cd), and development of prostate cancer. The objectives are: 1) to establish reliability of using formalin-fixed paraffin-embedded (FFPE) prostate tissue for analysis of Zn, Se and Cd tissue by comparing their levels in the fresh specimen; 2) to enhance the knowledge of prostate cancer environmental etiology by examining the association of Cd, Zn, Se with the development and progression of cancer on the basis of using FFPE prostate tissue specimen; 3) to enhance the use of the AFIP National Tissue Repository of archival tissues. Based on use of high-resolution inductively-coupled mass-spectrometry (ICP-MS), we developed a method for analysis of Zn, Cd, Se and also iron (Fe) in FFPE prostate. Our results demonstrated that in comparison with fresh tissue, in FFPE tissue the recoveries of Se, Fe, Cd and Zn were progressively decreased as 97±11%, 82±22%, 59±23% and 24±11%, respectively. Thus, the use of correction factors, determined as k=0.16 for Se, k=0.20 for Fe, k=0.27 for Cd and k=0.67 for Zn, is required to estimate the retrospective levels of these elements in the parental non-processed fresh (wet) prostate tissue. The developed methodology was applied to analyze the levels of Zn, Cd, Se and Fe in archival FFPE tissue specimens to study correlation between the metals' content and prostate cancer development.

15. SUBJECT TERMS
Prostate cancer, prostate-specific antigen, formalin-fixed paraffin-embedded tissue, trace metals, high-resolution inductively coupled mass-spectrometry

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	16	

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Project Progress Report

Introduction.

Prostate cancer is most frequently diagnosed form of cancer and the second leading cause of cancer death in USA men. A better understanding prostate cancer etiology, with focus on protective and aggravating factors may facilitate preventative or prospective measures to be taken to reduce morbidity and mortality from this disease. Three environmental trace elements, zinc (Zn), selenium (Se) and cadmium (Cd), have been highlighted in the literature in relation to the development of prostate disease (*1, 2*). Zinc (Zn) is second in abundance metal in human body that serves as cofactor of more than 300 enzymes participating in various biochemical reactions. In prostate, Zn is accumulated up to 10-fold higher levels (~150 μg/g) than in any other organ, and it is require for a healthy state of the gland (*3*), whereas several-fold decrease have been observed in the malignant tissue (*4-6*). Cadmium (Cd) possesses carcinogenic effect that is hormonally mediated (*7, 8*), and is recognized as a risk factor in development of prostate cancer (*2, 9-11*). In prostate, cadmium has demonstrated a distinct antagonist effect with Zn, presumably due to competing for binding sites with the same ligands in cell (*12, 13*). Selenium (Se) , a component of several amino acid (selenocysteine) and proteins (selenoproteins) (*14*), serves as antioxidant protectors (*15*) and is involved in DNA reparation (*16*). Studies have demonstrated an inverse correlation between environmental Se levels and prostate cancer mortality (*17, 18*) and established beneficial role of Se in reducing risk of cancer, where Se can interfere with carcinogenic activity of Cd (*4, 19-20*). The general purpose of the current project is to examine association between Zn, Se and Cd on the development of prostate cancer that remains unclear or under-researched.

The current investigation is an extension of our preliminary study (*21*). In current project, we developed the methodology for the analysis of levels of Zn, Se and Cd in formalin-fixed paraffin-embedded (FFPE) prostate tissue (objective 1), followed by applying this technology to the analysis of clinically and histologically well-characterized FFPE prostate tissue specimens to study correlations of these metals' levels with the development of cancer (objective 2).

Description of project accomplishment.

Initially, training was performed to master the technique of high resolution inductively coupled plasma mass-spectrometry analysis (ICP-MS) of trace metals in materials of different nature including biological tissues. During the training, the samples were prepared for analysis employing a digestion technique based on the use of microwave-accelerated digestion system (MARS-X and MARS-5 microwave ovens (CEM Corp., Matthews, NC) using nitric acid and hydroxide peroxide. The training was performed on ICP-MS machine Thermo Finnigan Element 2 (Thermo Electron Corp., Bremen, Germany).

Next, we explored possibility of using FFPE tissue to assess the levels of Cd, Zn and Se in the original fresh tissue. In this panel, it was also included an additional element, iron (Fe), as a catalyst facilitating generation of free radicals, and therefore hypnotized at the loci of its high deposition to contribute in prostate cancerogenesis. In these experiments, we used yttrium (Y) and ^{74}Se as internal standards and corresponding standard solutions of Fe, Se, Zn and Cd for quantification. One of the most important questions to be answered was if procedure of embedding tissue in paraffin leads to loss of the metals; therefore, we performed comparison of the metals' levels between FFPE tissue and the matching fresh tissue.

Establishing the instrumental parameters and the technique

Development of a methodology for the determination of Fe, Zn, Se and Cd in FFPE tissue was performed in a number of steps to optimize the major factors affecting the precision of the analysis. First, we established optimal parameters of ICP-MS technique and estimated the method detection limit (MDL) for each element (Tables 1 and 2) [30,31]. A number of studies have shown that the addition of carbon (in the form of glucose, glycerol, methanol, TRIS, acetonitrile, CH_4 etc.) to an ICP-MS sample increases signals of certain high ionization potential elements, including Se (*22-24*). In our experiments, the addition of acetic acid (AcOH) at 3% resulted in an increase of the Se signal 2.5-fold and 2-fold improvement in the detection limit, whilst the analysis of Fe, Zn and Cd was not affected. Thus, in further experiments, we used the addition of AcOH to the running solutions for ICP-MS.

Table 1. ICP-MS operating conditions

ICP operating parameters setting	
Cooling gas flow	16 $L\ min^{-1}$
Auxiliary gas flow	0.8 $L\ min^{-1}$
Sample gas flow	1.05 $L\ min^{-1}$
Additional gas flow	0.14 $L\ min^{-1}$
Plasma power	1550 *W*
Sampler/skimmers cones	Nickel
Mass spectrometer settings	
Resolution	>10000
Analytes	^{57}Fe, ^{66}Zn, ^{76}Se, ^{77}Se, ^{111}Cd
Internal standards	^{74}Se (for Se), ^{89}Y (for Fe, Zn, Cd)
Scan Type	E-scan
Mass window	100%
Integration window	20%
Runs	4
Passes	5
Dead time correction	6 *ns*

Table 2. Estimation of the method detection limits for Fe, Zn, Se and Cd

Parameters	Fe *(µg/L)*	Zn *(µg/L)*	Se *(µg/L)*	Cd *(µg/L)*
Instrument Detection Limit [a]	8.7	0.92	0.20	0.010
Analyzed with 3% of AcOH:				
Instrument Detection Limit	9.6	0.92	0.03	0.008
Generated concentration in solution	50.0	4.50	0.20	0.050
Determined concentration [b]	54.9 ±7.9	4.51 ±0.20	0.20 ±0.02	0.052 ±0.009
Method detection limit [c]	23.9	0.61	0.06	0.026

[b] - determined as 3xSD for blank samples (n=15)
[b] - the samples (n=15) were processed according to the method protocol
[c] - determined as 3xSD for the measured concentrations

Analysis of a standard reference material

At the second step, we tested the method analyzing a standard reference material, dried bovine liver (Cat. No 1577b purchased from National Institute of Standards and Technology, Gaithersburg, MD). . Initially, we analyzed ~150 mg of the tissue (a large scale preparation) that is close to the amount used by the manufacturer to certify the metals' concentrations. The samples (n=15) were digested with 70% nitric acid at 180^0 C and analyzed by ICP-MS. The determined levels of Fe, Zn, Se and Cd were found to be close to the certified values (within the range of SD, Table 3). In all experiments, we observed a slightly decreased Cd concentration in comparison with the certified value that most likely reflected the real Cd content in the used source container. Next, we analyzed the same material in a small scale preparation, matching the actual amount of tissue that can be recovered from a typical FFPE tissue preparation. About 10 mg of SRM (n=15) was digested using micro-vessels at 110^0 C and analyzed similarly. The determined metals' concentrations and the related relative standard deviation (RSD) values were found to be similar to those obtained in the former experiment (Table 3), indicating applicability of the small-scale digestion technique for analysis of the tissue specimens.

Table 3. Analysis of SRM (dried bovine liver) for Fe, Zn, Se and Cd levels by ICP-MS. Samples were prepared by digestions of the material either in a large or a small scale. For each series, the data were derived from two independent experiments. Concentrations are based on dry-weight tissue.

Analyzed element (content in tissue)	Certified value, RSD *(%)*		Large-scale preparation Determined value, RSD *(%)*		Large-scale preparation, (analyzed with 3%AcOH) Determined value, RSD *(%)*		Small-scale preparation, (analyzed with 3%AcOH) Determined value, RSD *(%)*	
Fe (mg/kg)	184 ±15	8	173 ±1	1	179 ±5	3	181 ±4	2
Zn (mg/kg)	127 ±16	13	118 ±3	3	122 ±4	3	125 ±5	4
Se (µg/kg)	730 ±60	8	735 ±35	5	723 ±14	2	700 ±13	2
Cd (µg/kg)	500 ±30	6	441 ±12	3	455 ±17	4	443 ±10	2

Analysis of the metals recovery from deparaffinized FFPE prostate tissue

Third, we assessed how the levels of Fe, Zn, Se and Cd in prostate tissue withstand the whole paraffin embedding/deparaffinization process. During this study we also optimized the technique for paraffin extraction. We assumed that this process does not markedly affect the metals levels in tissue, because most of their potential leakage from the tissue should have already occurred during the tissue embedding into paraffin.

Initially, we analyzed tissue taken from different areas of the same prostate gland. This specimen was obtained from a single case of radical prostatectomy performed for prostate cancer, taken from an area distant from a localized cancer focus and having no evidence of prostatic hypertrophy. The portions of the tissue (in triplicate analysis) were analyzed on dry weight basis directly (control), and after formalin fixation, embedding into paraffin and its removal using either automated or manual processing. Both techniques of paraffin extraction resulted in the detection of similar levels of the metals that had some differences in comparison with fresh tissue (Table 4). While the level of Se in FFPE tissue constituted 110±14% of that in the fresh tissue, the levels of Fe, Zn and Cd were 87±20%, 42±18% and 60±10%, respectively. The relatively high RSD values, compared with those obtained in analysis of the SRM were likely to be attributed to an uneven distribution of the metals within the gland. Indeed, the difference in their concentrations between the samples of fresh tissue was up to 3-fold. This experiment indicated that Se withstands the tissue embedding relatively well, whilst three other elements exhibit a certain leakage from the tissue.

Table 4. Levels of Fe, Zn, Se and Cd in samples of fresh and deparaffinized FFPE prostate tissue obtained from a single prostate gland specimen. For each sample it was analyzed 10-15 µg of dried tissue. Concentration values are based on dry-weight tissue.

Sample	Type of tissue processing	Fe *(mg/kg ±SD)*	Zn *(mg/kg ±SD)*	Se *(mg/kg ±SD)*	Cd *(µg/kg ±SD)*
1	Fresh tissue (control)	88 ±11	521 ±123	1.47 ±0.11	411 ±44
	FFPE-tissue, machine-based deparaffinization [a]	71 ±6	234 ±69	1.39 ±0.02	289 ±48
	FFPE-tissue, handling-based deparaffinization [b]	73 ±16	203 ±43	1.45 ±0.10	223 ±33
2	Fresh tissue (control)	88 ±5	716 ±390	1.53 ±0.23	422 ±147
	FFPE-tissue, machine-based deparaffinization	85 ±38	231 ±52	1.46 ±0.31	289 ±71
	FFPE-tissue, handling-based deparaffinization	95 ±13	404 ±119	1.84 ±0.27	241 ±44
3	Fresh tissue (control)	129 ±11	408 ±211	1.16 ±0.20	379 ±45
	FFPE-tissue, machine-based deparaffinization	84 ±19	327 ±126	1.52 ±0.42	216 ±19
	FFPE-tissue, handling-based deparaffinization	63 ±5	150 ±24	1.24 ±0.20	182 ±13
4	Fresh tissue (control)	86 ±13	692 ±77	1.30 ±0.08	336 ±35
	FFPE-tissue, machine-based deparaffinization	99 ±10	282 ±48	1.35 ±0.11	205 ±4
	FFPE-tissue, handling-based deparaffinization	84 ±5	483 ±147	1.56 ±0.20	195 ±10
5	Fresh tissue (control)	107 ±21	893 ±212	1.58 ±0.09	328 ±12
	FFPE-tissue, machine-based deparaffinization	89 ±10	414 ±132	1.54 ±0.12	215 ±31
	FFPE-tissue, handling-based deparaffinization	111 25	416 ±149	1.80 ±0.05	273 ±47
6	Fresh tissue (control)	107 ±16	1212 ±102	1.45 ±0.16	271 ±4
	FFPE-tissue, machine-based deparaffinization	118 ±15	281 ±61	1.74 ±0.16	182 ±25
	FFPE-tissue, handling-based deparaffinization	97 ±12	350 ±79	2.05 ±0.23	159 ±31
7	Fresh tissue (control)	124 ±23	855 ±271	1.35 ±0.15	358 ±36
	FFPE-tissue, machine-based deparaffinization	84 ±19	156 ±50	1.38 ±0.29	158 ±26
	FFPE-tissue, handling-based deparaffinization	90 ±28	209 ±43	1.33 ±0.20	160 ±17

[a] deparaffinization with xylene at 55^0 for 1h
[b] deparaffinization with hexane at 20^0 C for 1 week

In the final experiment, we compared levels of Fe, Zn, Se and Cd between fresh (dried) and matching FFPE prostate tissue specimens obtained from 15 subjects. Studies with human tissues were approved and conducted in accordance with the policies of the Institutional Review Boards of Armed Forces Institute of Pathology. The samples were received de-identified (coded) from the National Institute of Health (NIH)-sponsored Prostate Cancer Tissue Resource (www.prostatetissues.org) and provided by our co-investigator, Dr. André Kajdacsy-Balla (University of Illinois at Chicago). FFPE samples (in triplicates) were deparaffinized and analyzed altogether with the matching control samples as in the former experiment. The result of this analysis is shown in (Fig. 1 and 2.

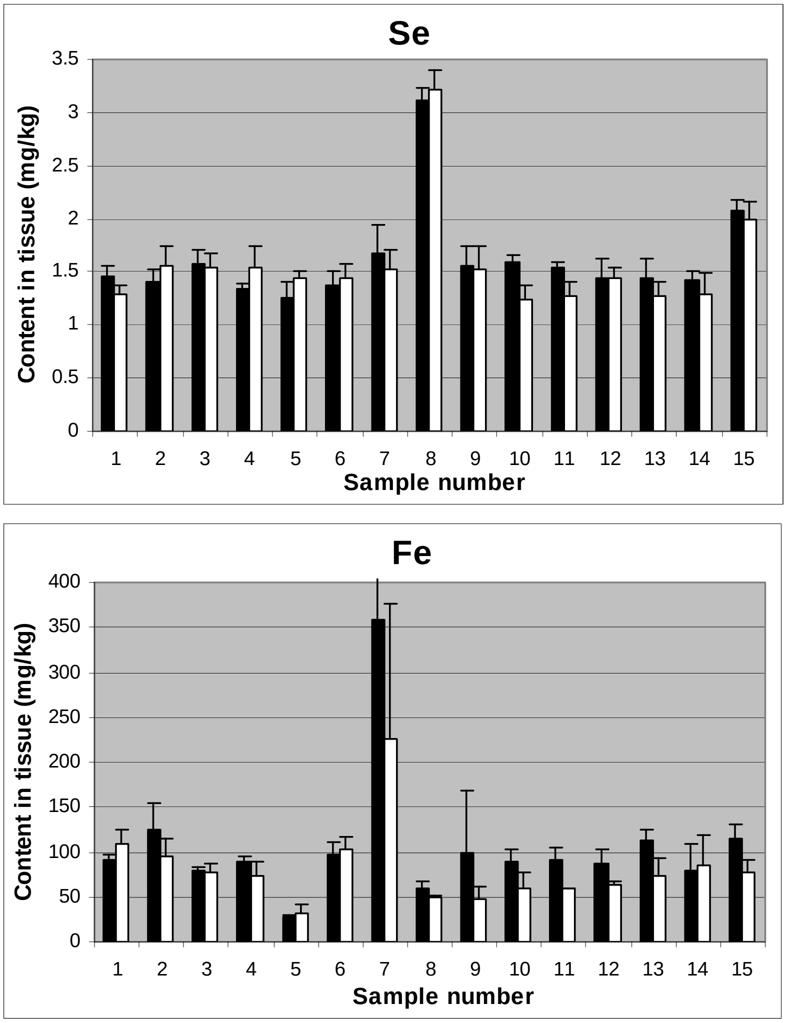

Fig. 1. Levels of Se and Fe in fresh and deparaffinized FFPE prostate tissue obtained from patients (n=15). The bars filled with black correspond to fresh tissue samples, the bars filled with white correspond to deparaffinized FFPE tissue samples. Error bars correspond to SD values. Concentrations are based on dry-weight tissue.

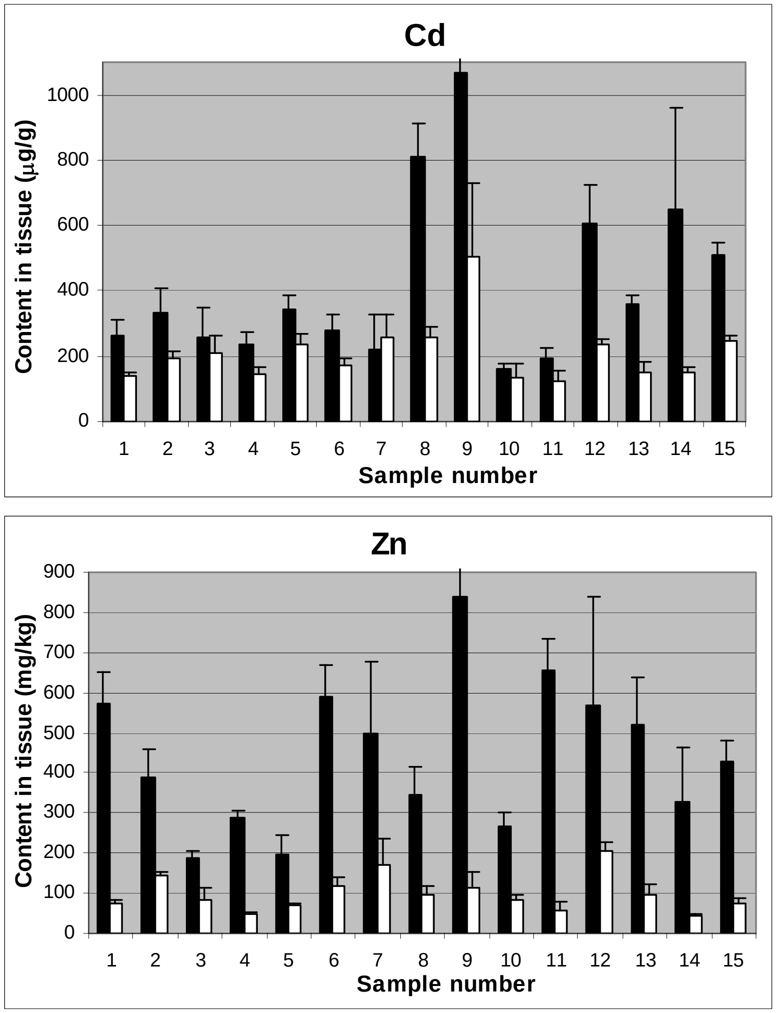

Fig. 2. Levels of Cd and Zn in fresh and deparaffinized FFPE prostate tissue obtained from different patients. The bars filled with black correspond to fresh tissue samples, the bars filled with white correspond to deparaffinized FFPE tissue samples. Error bars correspond to SD values. Concentrations are based on dry-weight tissue.

The resulting data were subjected to statistical analysis (Table 5). We found that the percentage recovery of the metals from the deparaffinized tissue (compared with the control tissue) was essentially the same as in the former experiment, where we analyzed serial aliquots of a single tissue sample. The average values were 82±22% for Fe, 97±11% for Se, 59±23% for Cd and 24±11% for Zn. The levels of Fe and Se in dried fresh and deparaffinized tissue were found to be highly correlated (r=0.86, $p<0.001$ and r=0.88, $p<0.001$, respectively, Table 5). A significant linear relationship was also seen for the Cd values (r=0.69, p=0.004). However, no significant linear relationship was observed between the Zn values (r=0.38, p=0.166) consistent with the highest difference in the distribution of Zn among these metals in prostate, observed in analysis of a single gland in the former experiment. Thus, Se, Fe, Cd and Zn withstand the tissue embedding into paraffin/deparaffinization in a different degree, ranging from excellent for Se to relatively low for Zn.

Table 5. Distribution of metal concentration values, Pearson correlation coefficients and slopes for fresh and deparaffinized FFPE prostate tissue samples obtained different individuals (n=15), based on the levels ranged by the results for the fresh tissue. Statistical significance was set at *P* less than 0.05. Significant differences at the p<0.01 level are marked with **.

Element	Range *(mg/kg)*		Median *(mg/kg)*		Log Mean *(mg/kg)*		SD *(µg/kg)*		Pearson Coeff.		
	fresh tissue	deparaff. tissue	fresh tissue	deparaff. tissue	fresh tissue	deparaff. tissue	fresh tissue	deparaff. tissue	(*r*)	r^2	*p*-value
Fe	27-359	31-226	90	74	93	74	1.7	1.6	0.861	0.741	<0.001**
Zn	187-840	45-204	427	82	408	90	1.6	1.6	0.377	0.142	0.166
Se	1.3-3.1	1.2-3.2	1.5	1.4	1.6	1.5	1.3	1.3	0.876	0.767	<0.001**
Cd	0.2-1.1	0.1-0.5	0.3	0.2	0.4	0.2	1.7	1.5	0.693	0.481	0.004**

Estimation of initial levels of the metals in fresh (non-processed) prostate tissue

The differential recovery of Se, Fe, Cd and Zn in FFPE prostate tissue requires using different correction formulas to estimate their initial content in the non-processed tissue. Based on the loss of tissue weight during the processing, consisting 82.8±1.2% for water and 1.2±0.3% for lipids known for the prostate (*25-27*), the original level of an element may be assessed via the equation:

$$C_{(FRESH)} = \{[100-(82.8+1.2)]/100 \times 100/P(\%)\} \times C_{(FFPE)}, \text{ or}$$

$$C_{(FRESH)} = 16/P(\%) \times C_{(FFPE)}, \text{ or}$$

$$C_{(FRESH)} = k \times C_{(FFPE)}$$

where $C_{(FRESH)}$ is the initial concentration of the element in the fresh tissue, $C_{(FFPE)}$ is its concentration in FFPE tissue, P(%) is the (pre-determined) percentage of recovery of this element in the FFPE tissue, and respectively, k_i=16/P(%) is the derivative correction factor. Based on determined recovery of Se, Fe, Cd and Zn, the corresponding factors represent the values of k_{Se}=0.16, k_{Fe}=0.20, k_{Cd}=0.27 and k_{Zn}=0.67, that allows to estimate these elements' retrospective content in the analyzed FFPE tissue.

Applying the developed methodology to the analysis of FFPE prostate tissue specimens.

Next we used the developed technique to the analysis of clinically and histologically well-characterized FFPE prostate tissue specimens (objective 2). Eighty subjects (forty pairs) were selected for a nested case-control study based on post-radical prostatectomy recurrence vs. non-recurrence. The selection of the samples was performed in the laboratory of André Kajdacsy-Balla (University of Illinois at Chicago). For each recurrence case, a non-recurrence case was matched for known risk factors as age, ethnicity, Gleason score, and a stage of the disease. All non-recurrence cases had at least 5 years of follow up, but recurrence cases did not have a time limitation. Thus, 80 samples were analyzed (in triplicates) by ICP-MS for the levels of Se, Fe, Cd and Zn as above (total, 240 sub-samples for the instrumental analysis). Currently, the processing of the obtained data (involving statistical analysis) is in progress.

Key research accomplishments.

On this investigation, we have developed method for simultaneous analysis of Zn, Cd, Se, and Fe in FFPE prostate tissue using sector-field (high-resolution) ICP-MS. We have shown that in comparison with fresh tissue, Se and Fe are retained in FFPE-tissue about at the original levels (~97% and 82%, respectively), whereas Cd and Zn levels are decreased (~59% and 24%, respectively). Thus, the corresponding correction factors should be applied to the determined levels of these metals in FFPE-tissue to assess their original content in the fresh tissue.

Reportable outcomes.

Abstracts, presentations and publications:

1. Sarafanov A., Todorov T., Kajdacsy-Balla A., Gray M., Macias V. and Centeno J. (2007) Analysis of iron, zinc, selenium and cadmium in paraffin-embedded prostate tissue specimens using inductively coupled plasma mass-spectrometry. *J. Trace Elem. Med. Biol. 2008* (in press).
2. Van der Voet G.B., Sarafanov A.G., Todorov T.I., Centeno J.A., Jonas W., Ives J., Mullick F.G. (2007) Clinical and analytical toxicology of dietary supplements: a case study and a review of the literature. *Biological Trace Element Research 2008.* (in press).
3. Sarafanov A., Todorov T., Kajdacsy-Balla A., Gray M., Macias V., and Centeno J. (2007) Analysis of iron, zinc, selenium and cadmium in paraffin-embedded prostate tissue specimens using inductively coupled plasma mass-spectrometry. Innovative Minds in Prostate Cancer Today Conference, September 5-8, 2007, Atlanta, GA.
4. Sarafanov A., Todorov T., Kajdacsy-Balla A., Gray M., Macias V., and Centeno J. (2007) Analysis of iron, zinc, selenium and cadmium in paraffin-embedded prostate tissue specimens using inductively coupled plasma mass-spectrometry. 10th Annual Force Health Protection Conference, August 5-10, 2007, Louisville, KY.
5. Todorov, T., Gray, M. A., Sarafanov A.G., Kadjacsy-Balla, A., and Centeno, J. A. Comparison between the cadmium, zinc, selenium, iron and arsenic content in fresh and paraffin embedded tissue specimen. European Conference on Plasma Chemistry, Feb 18-23, 2007, Taormina, Italy.
6. Todorov, T., Gray, M. A., Kadjacsy-Balla, A., Mullick, F. G., and Centeno, J. A. Cadmium, zinc, selenium and arsenic in fresh and paraffin embedded prostate tissues, pp 143, Abstract #O-103. 9th International Symposium on Metal Ions in Biology and Medicine, May 21-24, 2006, Lisboa, Portugal.
7. Gray, M. A., Centeno, J. A., Todorov, T., Slaney, D. P., and Nacey, J. N. Environmental exposure to cadmium, zinc and selenium and risk of prostate cancer, pp 61, Abstract #O-21. 9th International Symposium on Metal Ions in Biology and Medicine, May 21-24, 2006, Lisboa, Portugal.
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Conclusion:

In this investigation we have developed a method for the accurate and precise determination of Zn, Cd, Se, and Fe in formalin-fixed paraffin-embedded prostate tissue. We found that in FFPE tissue, the recoveries of Se, Fe, Cd and Zn were progressively decreased as 97±11% (r=0.88), 82±22% (r=0.86), 59±23% (r=0.69) and 24±11% (r=0.38), respectively. Thus, the use of correction factors, determined as k=0.16 for Se, k=0.20 for Fe, k=0.27 for Cd and k=0.67 for Zn, is required to estimate the retrospective levels of these elements in the parental non-processed fresh (wet) prostate tissue. The technique enables the analysis of archival FFPE prostate tissue for the concentrations of Fe, Zn, Se and Cd to study association between the levels of these metals and prostate disease.

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